UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	: Linda H. Malkas
By the Examiner	: Huff, Sheela Jitendra
Docket No.	: 80371/5
Serial No.	: 10/083,576
Filed	: February 27, 2002
Group Art Unit	: 1643
Title	: METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Dear Sir:

I, R. Christopher Rueppell, declare as follows:

1. I am a registered patent attorney (#47,045) and an attorney of record in the above-identified patent application.

2. Pamela E. Bechtel is a co-inventor of the subject matter of the above-identified patent application.

3. Pamela E. Bechtel received a Bachelor of Science degree in 1993 from Pennsylvania State University. In 1998, Pamela E. Bechtel was awarded the degree of Doctor of Philosophy in Pharmacology and Toxicology from the University of Maryland.

4. Pamela E. Bechtel's doctoral dissertation entitled: PROLIFERATING CELL NUCLEAR ANTIGEN IN MALIGNANCY, was submitted to the Faculty of the Graduate School of the University of Maryland in 1998 and was approved May 8, 1998. Pamela E. Bechtel's doctoral dissertation evidences the identification of the presence of a cancer specific isoform of proliferating cell nuclear antigen (csPCNA) in various malignant cell lines, including malignant prostate cells, colon cells, brain and cervical cells, and leukemia cells.

5. The above-identified patent application as originally filed includes the following:

The source of the tissue or body fluid is from a subject afflicted with a cancer. The particular cancer is not critical to the present invention. The cancers can be carcinomas, sarcomas, lymphomas, or leukemias. Examples of such cancers include cervical carcinoma, mammary gland carcinoma of ductal or lobular origin, gliomas, prostate, lung, esophageal, stomach, and ovarian cancer.

(Page 10, Lines 10-17). The above-identified patent application does state that the csPCNA isoform exists in malignant cell types and, as shown above, discloses various malignant cell types within which the csPCNA isoform exists.

6. FIGS. 9, 10, 11, and 12 (Exhibit A), originally submitted in the Response filed November 23, 2005 (re-submitted in Response to Non-Compliant Amendment filed January 6, 2006) for the above-identified patent application are FIGS. 1, 2, 3, and 4, respectively, found on pages 101, 102, 103, and 104, respectively, of Pamela E. Bechtel's doctoral dissertation. Reproductions of pages 101, 102, 103, and 104 of Pamela E. Bechtel's doctoral dissertation are found in Exhibit B. FIGS. 9, 10, 11, and 12 were submitted to overcome a §112, first paragraph rejection.

7. The Amendments to the Specification (Exhibit C) submitted in the Response filed November 23, 2005 for the above-identified patent application with respect to FIGS. 9, 10, 11, and 12 were based on the disclosure from pages 88-89 and 99-100 of Pamela E. Bechtel's doctoral dissertation. Reproductions of pages 88-89 and 99-100 of Pamela E. Bechtel's doctoral dissertation are found below and in Exhibit D. These Amendments to the Specification were submitted to overcome a §112, first paragraph rejection.

8. FIGS. 9, 10, 11, 12 and the Amendment to Specification submitted in the Response filed on November 23, 2005 and the Response filed January 6, 2006 for the above-identified patent application properly support the disclosure of the presence of the cancer specific Proliferating Cell Nuclear Antigen (csPCNA) in various malignant cell lines, as originally disclosed in the above-identified patent application.

9. The **Results** from Pamela E. Bechtel's doctoral dissertation that support the identification of the presence of csPCNA in malignant cells regardless of cell line are reported below, the experimental methods employed in support (pages 86-87 of Pamela E. Bechtel's doctoral dissertation) were:

METHODS

Cell Culture: HeLa cells were maintained in Dulbucco's Modified Eagles Medium (DMEM) with 10% bovine calf serum. HL60 and LNCaP cells were maintained in RPMI 1640 media with 10% fetal bovine serum. PC50 cells were maintained in Ham's F12K media with 7% fetal bovine serum. KGE90, KYE350 and SW48 cells were maintained in L-15 medium with 10% bovine calf serum. T98 cells were maintained in Eagles MEM with nonessential amino acids, 1.0 mM Sodium pyruvate and Earles BSS, and 10% fetal bovine serum.

DNA synthesome isolation: The DNA synthesome was isolated form LNCaP, PC50, KGE90, KYE350, HL60, HeLa, T98 and leukemia cell pellets according to published procedures (Coll et al., 1996) with minor modifications. Briefly, cell pellets were Dounce homogenized in a buffer containing 200 mM sucrose, 50 mM Hepes, 5mM KCl, 2mM DTT and 0.1 mM PMSF and centrifuged at 2500 rpm for 10 min to remove cell debris and pellet the nuclei. EDTA and EGTA were added to a final concentration of 5mM to the supernatant. One volume of nuclear extraction buffer (350 mM KCl, 50 mM Hepes, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.1 mM PMSF) was added to the pellet and rocked at 4°C for 2hr. The supernatant was centrifuged at 18,000 rpm for 3 min. The supernatant was removed and centrifuged at 60,000 rpm for 22 min. The resulting supernatant was collected (S3 fraction). The nuclear pellet was centrifuged at 22,000 rpm for 3 min. The supernatant was removed and centrifuged at 60,000 rpm for 15 min. The resulting supernatant (NE fraction) was combined with the S3 fraction, layered on top of a 2 M sucrose cushion and centrifuged overnight at 40,000 rpm. The synthesome fraction was collected for analysis.

2 Dimensional polyacrylamide gel electrophoresis (2D PAGE): DNA synthesome protein (20-40 μg) was loaded onto the first dimension tube gel (9.2 M urea, 4% acrylamide, 2% ampholytes (pH 3-10), and 20% Triton X-100). The polypeptides were separated along a pH gradient established using 100 mM NaOH and 10 mM H_3PO_4. The tube gels were placed onto an 8% acrylamide SDS gel, and the polypeptides were resolved by molecular weight. The proteins were then transferred electrophoretically to nitrocellulose membranes.

Western blot analysis: An antibody directed against PCNA (PC 10 Oncogene Science) was used at a dilution of 1:1000. Immunodetection of PCNA was performed using a light enhanced chemiluminescence system (Amersham).

Leukemia samples: Chronic myelogenous leukemia (CML) samples were obtained from Dr. Moshe Talpaz through a collaboration with the MD Anderson Cancer Center. The acute myelogenous leukemia (AML) sample was obtained from Dr. Lynn Abruzzo through a collaboration with the Greenebaum Cancer Center.

10. PCNA in Malignant Prostate Cells (**Results**). The DNA synthesome was isolated from LNCaP and PC50 prostate adenocarcinoma cell lines and the components of the synthesome were resolved by two dimensional gel electrophoresis (2D PAGE). Western blot analysis was performed using an antibody directed against PCNA to determine whether the acidic form of PCNA was present in the malignant prostate cells. The acidic form of PCNA was readily detected in both of the prostate cancer cell lines examined (Figure 1).

(Figure Legend) Figure 1: The Protein migration pattern of PCNA from malignant prostate cells. The DNA synthesome was isolated from LNCaP and

PC50 prostate cancer cells. The components of the synthesome were resolved by 2D PAGE, and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility of PCNA is shown for (A) LNCaP cells and (B) PC50 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 1 (Exhibit B) from Pamela E. Bechtel's doctoral dissertation is similar to the FIG. 9 (Exhibit A) submitted in the Response (November 23, 2005 and January 6, 2006). The written description from Pamela E. Bechtel's doctoral dissertation regarding FIG. 1 (Exhibit D) is similar to the written description submitted in the Response with respect to FIG. 9 (Exhibit C). Thus, FIG. 9 and the written description associated with FIG. 9 provided in the Response filed on November 23, 2005 and January 6, 2006 for the above referenced application is supported by the above disclosure (reproduced in Exhibit B) in reference to Figure 1 of Pamela E. Bechtel's doctoral dissertation.

11. PCNA in Malignant Colon Cells (**Results**). PCNA associated with the DNA synthesome was isolated from three colon adenocarcinoma cell lines, KGE90, KYE350 and SW48. The components of the synthesome were resolved by 2D PAGE followed by Western blot analysis using an antibody directed against PCNA. The results of this analysis indicate that the three colon cancer cell lines contained the acidic form of PCNA (Figure 2). Two of the cell lines (KGE90 and KYE350) only contained the acidic form of PCNA while the SW48 cell line contained both forms of PCNA.

(Figure Legend) Figure 2: The protein migration pattern of PCNA from malignant colon cells. The DNA synthesome was isolated from KGE90, KYE350 and SW48 colon cancer cells. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) KGE90 cells, (B) KYE350 cells and (C) SW48 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 2 (Exhibit B) from Pamela E. Bechtel's doctoral dissertation is similar to the FIG. 10 (Exhibit A) submitted in the Response (November 23, 2005 and January 6, 2006). The written description from Pamela E. Bechtel's doctoral dissertation regarding FIG. 2 (Exhibit D) is similar to the written description submitted in the Response with respect to FIG. 10 (Exhibit C). Thus, FIG. 10 and the written description associated with FIG. 10 provided in the Response filed on November 23, 2005 and January 6, 2006 for the above referenced application is supported by the above disclosure (reproduced in Exhibit B) in reference to Figure 2 of Pamela E. Bechtel's doctoral dissertation.

12. PCNA in Malignant Brain and Cervical Cells (**Results**). PCNA was isolated form a malignant glioma cell line (T98) and a malignant cervical cancer cell line (HeLa) to determine whether the altered form of PCNA was present. 2D PAGE and Western blot analyses were performed using a PCNA specific antibody. Figure 3A shows that the altered form of PCNA was present in HeLa cells. Figure 3B illustrates that T98 cells contained the acidic form of PCNA.

(Figure Legend) Figure 3: The protein migration pattern of PCNA from malignant brain and cervical cells. The DNA synthesome was isolated from malignant glioma (t98) and cervical (HeLa) cells. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) HeLa cells and (B) T98 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 3 (Exhibit B) from Pamela E. Bechtel's doctoral dissertation is similar to the FIG. 11 (Exhibit A) submitted in the Response (November 23, 2005 and January 6, 2006). The written description from Pamela E. Bechtel's doctoral dissertation regarding FIG. 3 (Exhibit D) is similar to the written description submitted in the Response with respect to FIG. 11 (Exhibit C). Thus, FIG. 11 and the written description associated with FIG. 11 provided in the Response filed on November 23, 2005 and January 6, 2006 for the above referenced application is supported by the above disclosure (reproduced in Exhibit B) in reference to Figure 3 of Pamela E. Bechtel's doctoral dissertation.

13. PCNA in Leukemia **(Results)**. Blood samples from patients with chronic myelogenous leukemia (CML) and acute myelogenous leukemia (AML), serum collected form cancer free individuals, and a human leukemia cell line (HL60) were analyzed to determine whether the acidic form of PCNA was present. The DNA synthesome was isolated form the leukemia samples and the HL60 cell line and the components resolved by 2D PAGE followed by Western blot analysis using an antibody directed against PCNA. The protein migration pattern exhibited by PCNA is shown in Figure 4. The acidic form of PCNA was identified in HL60 cells (Figure 4A), the AML sample (Figure 4B) and all of the CML samples (Figures 4C-E). The acidic form of PCNA was not detectable in the serum collected form cancer free individuals.

(Figure Legend) Figure 4: The protein migration pattern of PCNA from leukemia cells. The DNA synthesome was isolated from HL60 cells, CML samples, and AML sample and serum collected form cancer free individuals. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) HL60 cells, (B) AML cells and (C-E) CML cells. The arrow indicates the position of the acidic from of PCNA.

Figure 4 (Exhibit B) from Pamela E. Bechtel's doctoral dissertation is similar to the FIG. 12 (Exhibit A) submitted in the Response (November 23, 2005 and January 6, 2006). The written description from Pamela E. Bechtel's doctoral dissertation regarding FIG. 4 (Exhibit D) is similar to the written description submitted in the Response with respect to FIG. 12 (Exhibit C). Thus, FIG. 12 and the written description associated with FIG. 12 provided in the Response filed on November 23, 2005 and January 6, 2006 for the above referenced application is supported by the above disclosure (reproduced in Exhibit B) in reference to Figure 4 of Pamela E. Bechtel's doctoral dissertation.

14. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false

statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

DATE: 8/16/06

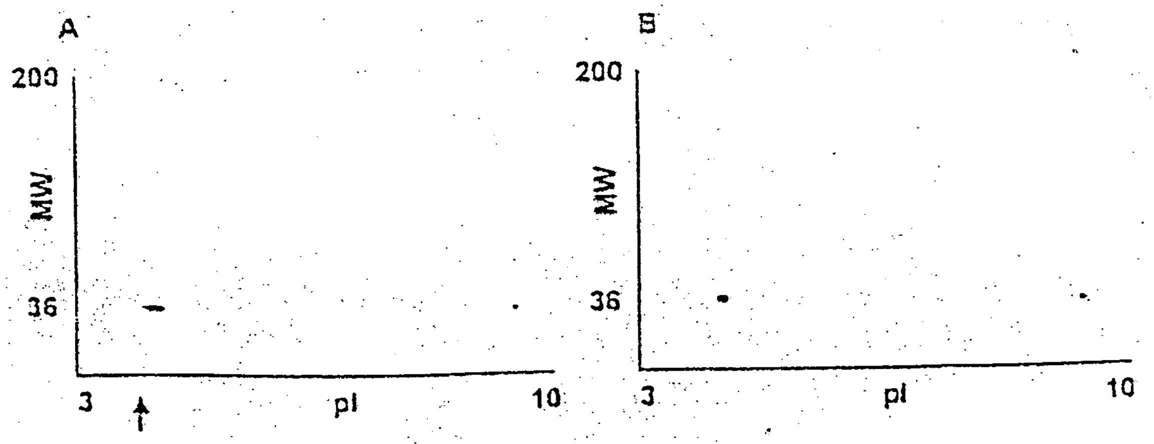
R. Christopher Rueppell
Attorney for Applicant
Registration No. 47,045

EXHIBIT A

1. FIGS. 9, 10, 11, and 12 as presented in the Response to the Notice of Non-Compliant Amendment filed January 6, 2006 pertaining to the Response filed November 23, 2005.

NEW SHEET

FIGURE 9



NEW SHEET

FIGURE 10

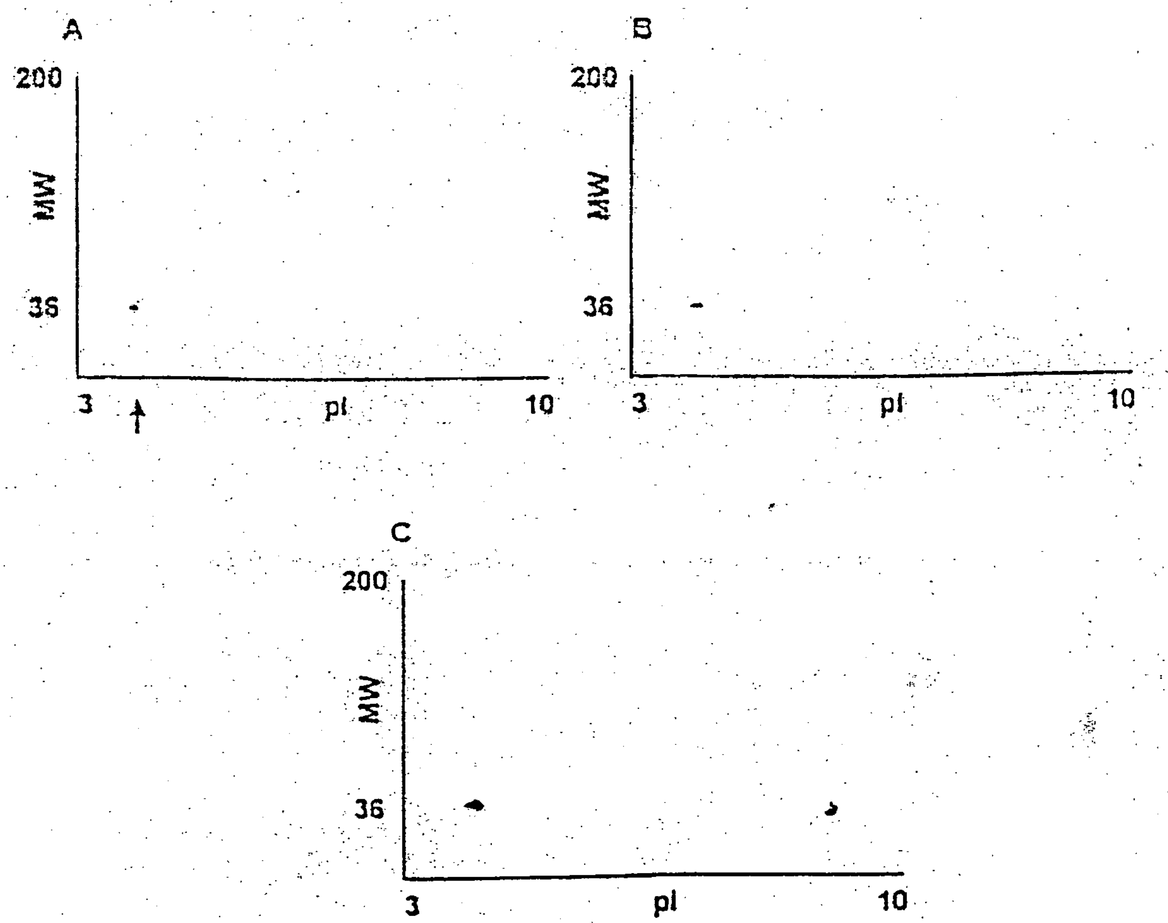
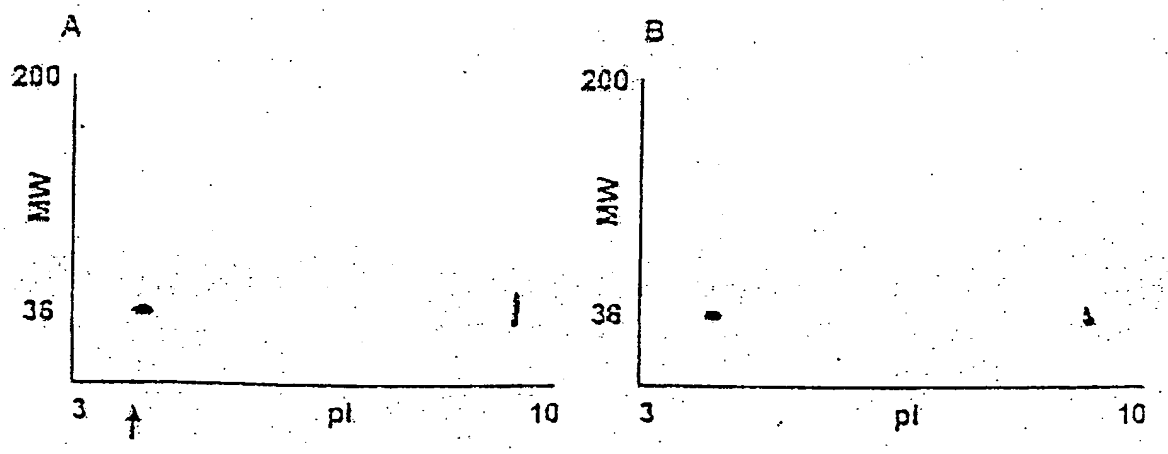


FIGURE 11



NEW SHEET

FIGURE 12

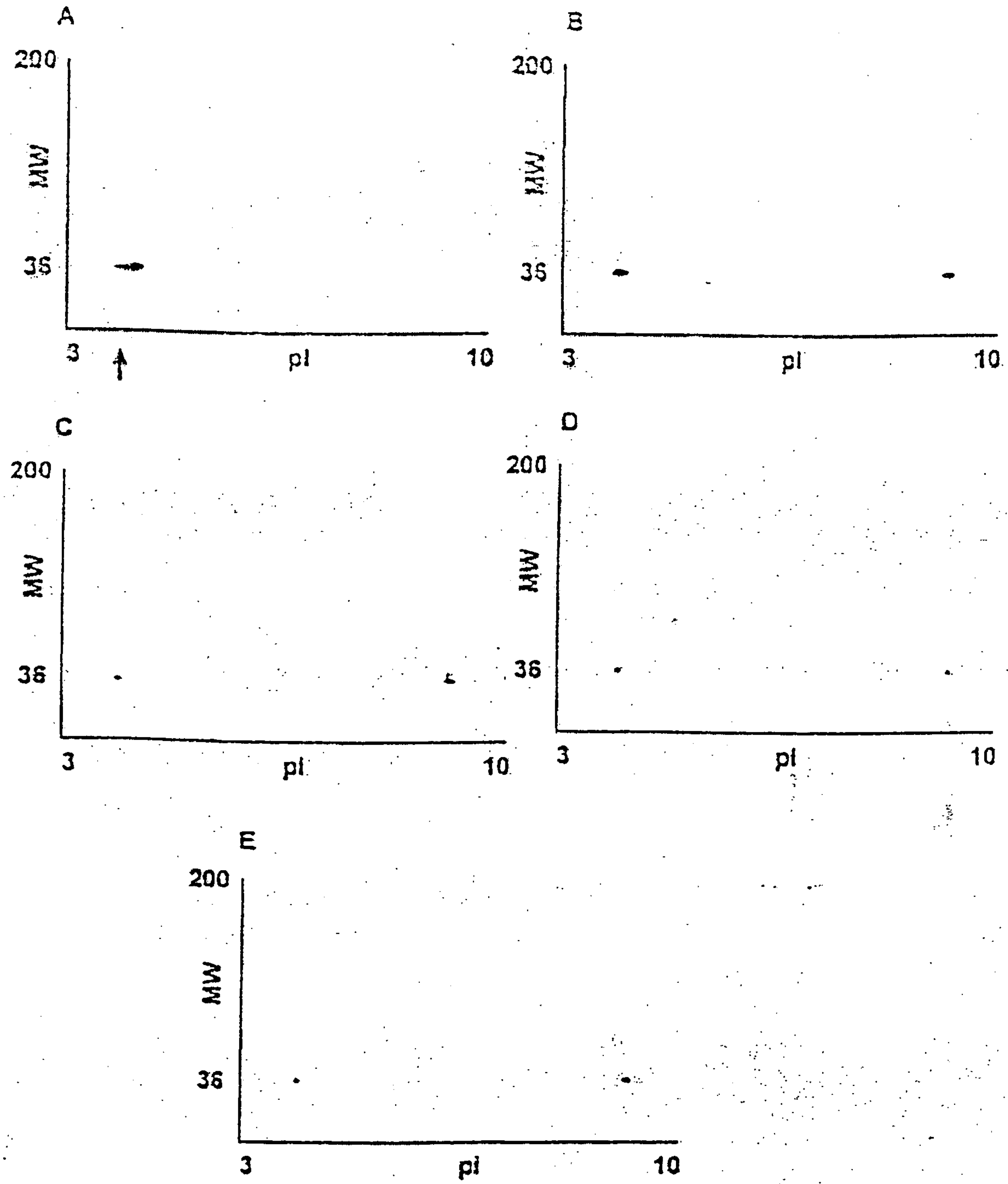


EXHIBIT B

1. Pages 101, 102, 103, and 104, evidencing Figures 1, 2, 3, and 4, of Pamela E. Bechtel's Doctoral Dissertation, approved on May 8, 1998.

Figure 1

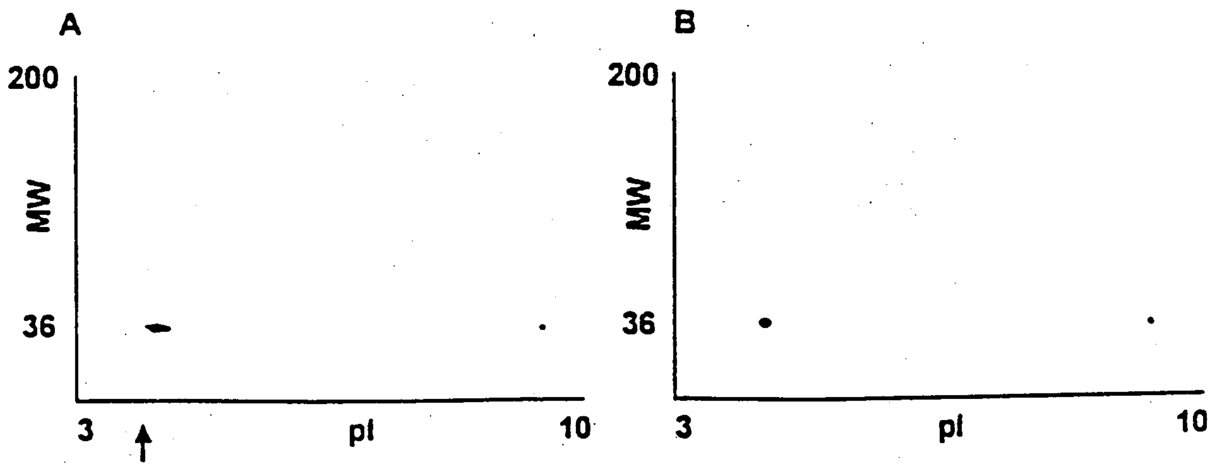


Figure 2

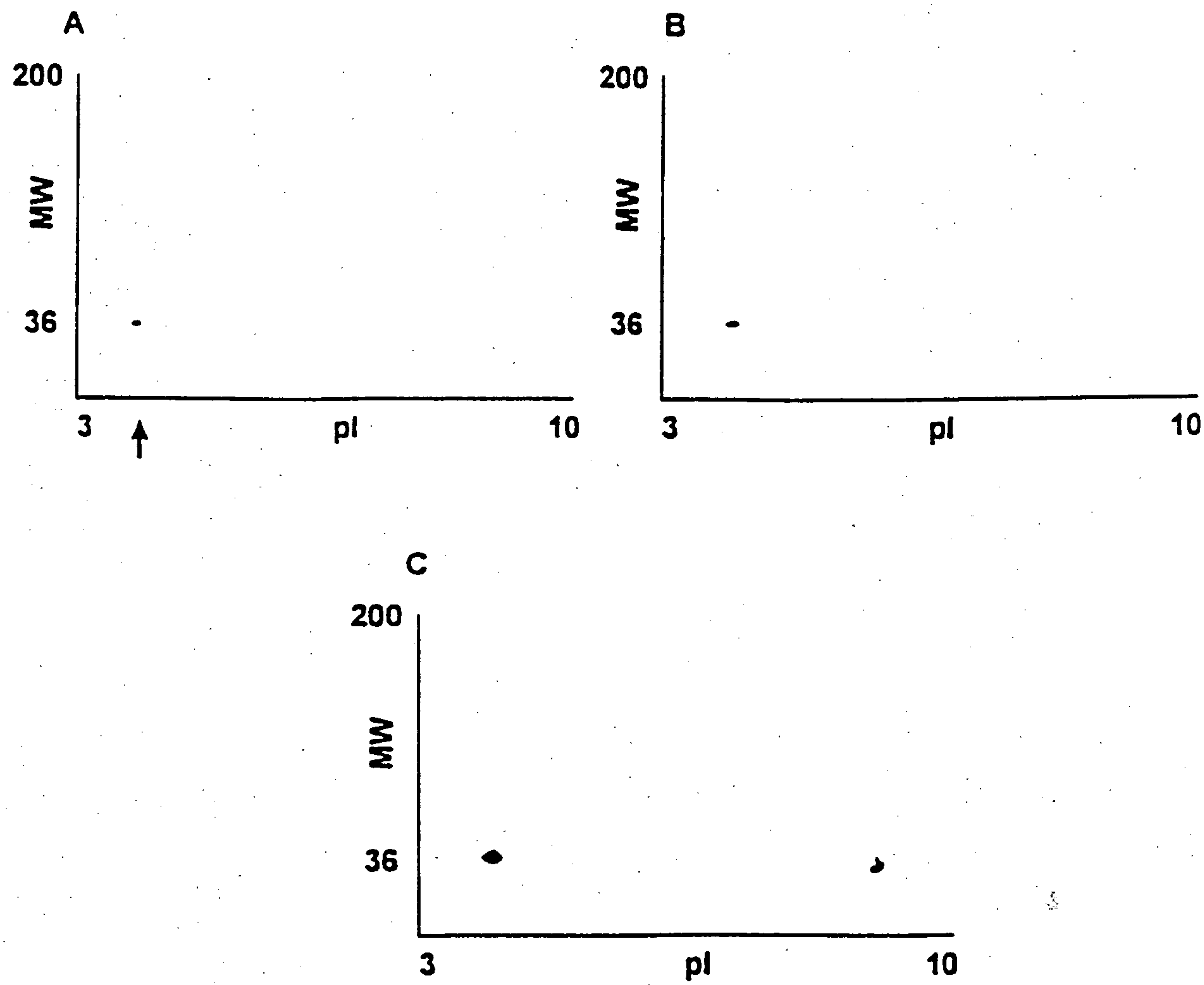


Figure 3

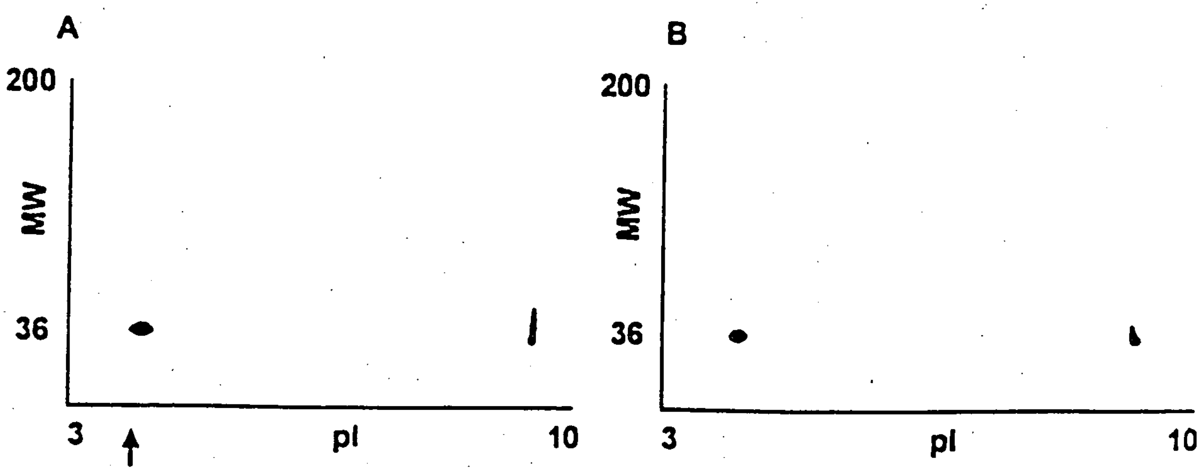
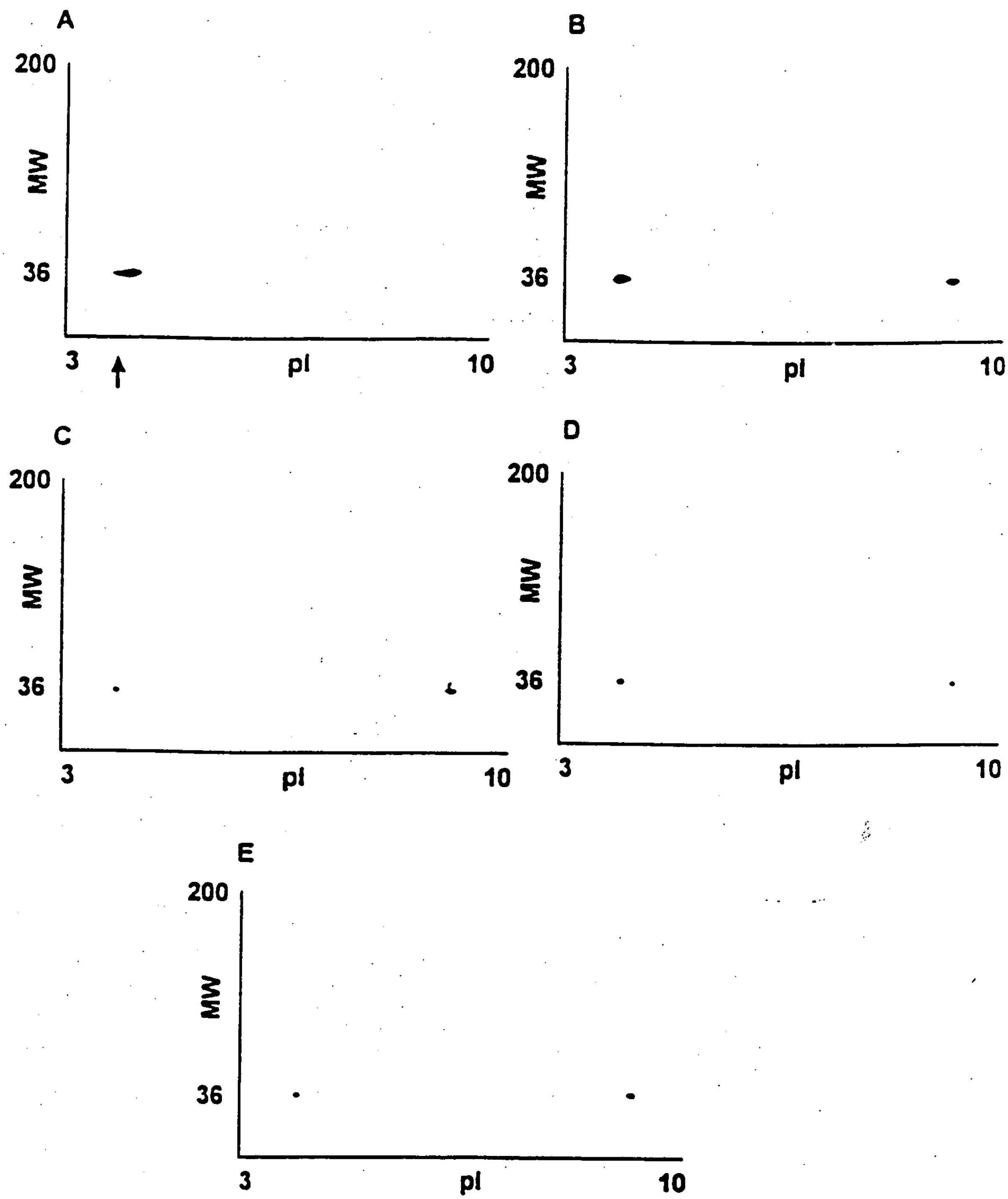


Figure 4


A
200
MW
36
3
pI
10
B
200
MW
36
3
pI
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C
200
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36
3
pI
10
D
200
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E
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EXHIBIT C

1. Amendments made to the Specification as submitted in the Response filed November 23, 2005.

AMENDMENT

In the Specification

Please amend the specification as follows:

In the BRIEF DESCRIPTION OF THE DRAWINGS section please add the following after "Figure 8" (Page 7, Lines 25-27):

Figure 9 is an illustration showing the presence of csPCNA in two different prostate adenocarcinoma cell lines. Western blot analysis of PCNA resolved by two-dimensional gel electrophoresis (2D-PAGE) from the LNCaP (A) and PC50 (B) cell lines, such that the acidic csPCNA isoform may be readily detected as indicated by the arrow in (A).

Figure 10 is an illustration showing the presence of csPCNA in malignant colon cells. The resolution of protein isolated from three malignant colon adenocarcinoma cell lines KGE90 (A), KYE350 (B), and SW48 (C) are shown below showing the presence of csPCNA (arrow).

Figure 11 is an illustration showing the presence of csPCNA in malignant cervical and brain cells. The HeLa malignant cervical cell line (A) and T98 malignant glioma cell line (B) were tested for the presence of csPCNA and both were found to contain the isoform, which were indistinguishable between the cancers.

Figure 12 is an illustration showing the presence of csPCNA in leukemia cells. The human leukemia cell line HL60 (A), leukemic cells obtained from the serum of an individual with acute myelogenous leukemia (AML) (B), and leukemic cells isolated from three individuals with chronic myelogenous leukemia (CML) (C-E) all express the csPCNA isoform (arrow).

Please insert the following on Page 10 after the second full paragraph that ends on Line 17 and before the next paragraph that begins on Line 18.

The cancer-specific isoform of proliferating cell nuclear antigen (csPCNA) is present in various cancers. Detection of the acidic csPCNA isoform for two different prostate adenocarcinoma cell lines is shown in FIG. 9. The position or resolution of the acidic csPCNA isoform may be readily detected as indication by the arrow in (A). FIG. 10 shows the detection of the acidic csPCNA isoform in malignant colon adenocarcinoma cell lines. Interestingly, two of the colon cell lines (KGE90 and KYE350) apparently contain only the csPCNA isoform. The resolution of csPCNA in these colon cels are identical to that of prostate and breast cells. Malignant cervical and brain cells also express the acidic csPCNA isofom, as shown in FIG. 11. The position of the csPCNA in the gels shown in FIG. 11 is indicated with an arrow and is identical to csPCNA observed in the other cancer cell lines. FIG. 12 illustrates the expression of the acidic csPCNA isoform in various leukemia cells. From these various examples, it has been shown that the csPCNA isoform resolves at exactly the same isoelectric point (pI) in all of the samples and represents a single distinct isoform. Therefore, the csPCNA isoform represents a general biomarker for the detection of malignancy, regardless of the type of cancer.

EXHIBIT D

1. Pages 88, 89, 99 and 100, evidencing the source of the Amendment to the Specification submitted in the Response filed November 23, 2005, of Pamela E. Bechtel's Doctoral Dissertation, approved on May 8, 1998.

RESULTS

PCNA in Malignant Prostate Cells

The DNA synthesome was isolated from LNCaP and PC50 prostate adenocarcinoma cell lines and the components of the synthesome were resolved by two dimensional gel electrophoresis (2D PAGE). Western blot analysis was performed using an antibody directed against PCNA to determine whether the acidic form of PCNA was present in the malignant prostate cells. The acidic form of PCNA was readily detected in both of the prostate cancer cell lines examined (Figure 1).

PCNA in Malignant Colon Cells

PCNA associated with the DNA synthesome was isolated from three colon adenocarcinoma cell lines, KGE90, KYE350 and SW48. The components of the synthesome were resolved by 2D PAGE followed by Western blot analysis using an antibody directed against PCNA. The results of this analysis indicate that the three colon cancer cell lines contained the acidic form of PCNA (Figure 2). Two of the cell lines (KGE90 and KYE350) only contained the acidic form of PCNA while the SW48 cell line contained both forms of PCNA.

PCNA in Malignant Brain and Cervical Cells

PCNA was isolated from a malignant glioma cell line (T98) and a malignant cervical cancer cell line (HeLa) to determine whether the altered form of PCNA was present. 2D PAGE and Western blot analyses were performed using a PCNA specific antibody. Figure 3A shows that the altered form of PCNA was present in HeLa cells. Figure 3B illustrates that T98 cells contained the acidic form of PCNA.

PCNA in Leukemia

Blood samples from patients with chronic myelogenous leukemia (CML) and acute myelogenous leukemia (AML), serum collected from cancer free individuals, and a human leukemia cell line (HL60) were analyzed to determine whether the acidic form of PCNA was present. The DNA synthesome was isolated from the leukemia samples and the HL60 cell line and the components resolved by 2D PAGE followed by Western blot analysis using an antibody directed against PCNA. The protein migration pattern exhibited by PCNA is shown in Figure 4. The acidic form of PCNA was identified in HL60 cells (Figure 4A), the AML sample (Figure 4B) and all of the CML samples (Figures 4C-E). The acidic form of PCNA was not detectable in the serum collected from cancer free individuals.

DISCUSSION

The diagnosis of cancer during its earliest stages of development is important in reducing cancer related mortality. Currently, a major limitation in the diagnosis of most cancers is the inability to reliably detect tumors prior to the onset of clinical manifestations. The identification of specific cellular transformation events that occur during the initial phase of tumor development may be beneficial in identifying novel tumor markers to better detect and monitor malignancies. A specific molecular alteration was recently identified in breast cancer cells which has the potential to serve as a breast cancer tumor marker (Bechtel *et al.*, submitted). To determine whether the altered form of PCNA was also present in other types of cancer cells, several different human malignancies were evaluated.

FIGURE LEGENDS

Figure 1: The protein migration pattern of PCNA from malignant prostate cells. The DNA synthesome was isolated from LNCaP and PC50 prostate cancer cells. The components of the synthesome were resolved by 2D PAGE, and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility of PCNA is shown for (A) LNCaP cells and (B) PC50 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 2: The protein migration pattern of PCNA from malignant colon cells. The DNA synthesome was isolated from KGE90, KYE350 and SW48 colon cancer cells. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) KGE90 cells, (B) KYE350 cells and (C) SW48 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 3: The protein migration pattern of PCNA from malignant brain and cervical cells. The DNA synthesome was isolated from malignant glioma (T98) and cervical (HeLa) cells. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) HeLa cells and (B) T98 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 4: The protein migration pattern of PCNA from leukemia cells. The DNA synthesome was isolated from HL60 cells, CML samples, an AML sample

and serum collected from cancer free individuals. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) HL60 cells, (B) AML cells and (C-E) CML cells. The arrow indicates the position of the acidic form of PCNA.